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The role of *Mn-SOD* and *Fe-SOD* genes in the response to low temperature in *chs* mutants of *Arabidopsis*

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Abstract: To determine whether the expression of iron superoxide dismutase (*Fe-SOD*) and manganese superoxide dismutase (*Mn-SOD*) increase superoxide-scavenging capacity, and thereby improve the survival rate of chilling sensitive (*chs*) mutants of *Arabidopsis*, 4 *chs* mutant (*chs1-1*, *chs1-2*, *chs2-1*, and *chs2-2*) and wild-type plants were grown under low (chilling, 13 °C; cold, 4 °C) and normal growth (23 °C) temperatures. Photosynthetic parameters were investigated following treatment with chilling, cold, and normal growth conditions. Chlorophyll content and maximum quantum efficiency of PSII primary photochemistry (Fv/Fm) were reduced in plants grown at chilling stress. The degree of chilling sensitivity of *chs1* mutant plants was significantly greater than that of wild-type and *chs2* mutants, as measured by chlorophyll fluorescence value and chlorophyll content. *MSD1* was expressed during chilling stress in *chs* mutant and WT plants, while expression of *FSD2* and *FSD3* SODs in *chs* mutants was not detected during any of the treatments. Our results suggest that *MSD1* expression in *chs1*-chilled plants responds to chilling, and that the lack of expression of *FSD2* and *FSD3* genes in *chs* mutants grown at chilling temperature supports the hypothesis that chloroplasts might be damaged, due to the *chs* mutation, when they are chilled.

Key words: *Arabidopsis thaliana*, chlorophyll content, *chs* mutant, *Fe-SOD*, Fv/Fm, *Mn-SOD*

1. Introduction

Plants have different resistance to chilling and freezing temperatures (Levitt, 1980). Freezing is lethal to most cellular organisms (Mutlu et al., 2013). In chilling-sensitive plants, oxidative stress is a significant component of chilling stress (Hodges et al., 1997). Chilling temperatures enhance the production of ROS. ROS, such as superoxide ($O2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2) react very rapidly with cellular macromolecules and produce damaging effects on DNA, lipids, and proteins (Van Breusegem et al., 1999a). To regulate the intracellular concentrations of ROS, plant cells have developed defense systems involving both enzymatic and nonenzymatic mechanisms (Alscher et al., 2002). Enzymes involved in selective detoxification include superoxide dismutases (SODs), which constitute the first line of cellular defense against ROS and catalyze the conversion of superoxide to H_2O_2 and O_2^-, catalase (CAT), which removes hydrogen peroxide (Bowler et al., 1992), and ascorbate peroxidase (APX) and glutathione reductase (GR) which scavenge H_2O_2 during the ascorbate-GSH cycle (Hammond-Kosack and Jones, 1996). To date, 4 types of SOD have been identified, and these metalloenzymes differ by the active site metal cofactor (Mn, Fe, Cu-Zn, or Ni) (Scandalios, 1997). *Mn-SOD* and *Fe-SOD* are localized to mitochondria and chloroplasts, respectively, whereas *Cu-ZnSOD* is present in the cytosol and chloroplasts (Bowler et al., 1992). Ni-dependent SOD, not found in plants, is completely unrelated to the better-known Cu-Zn-, Fe-, and *Mn-SOD*s (Youn et al., 1996). There are 7 SOD genes in the genome of *Arabidopsis* including 3 isoforms of both *Fe-SOD* (*FSD1*, *FSD2*, and *FSD3*) and *Cu-ZnSOD* (*CSD1*, *CSD2*, and *CSD3*) and a single isoform of *Mn-SOD* (*MSD1*) (Kliebenstein et al., 1998). A proteomic analysis (Zybailov et al., 2008) revealed the presence of *FSD1* in the chloroplast stroma, with the highest expression level in the rosette leaves of mature plants (Kliebenstein et al., 1998). The plastid localization of *FSD2* and FSD3 was confirmed using GFP fusions. *FSD2* and *FSD3* play key roles in early chloroplast development (Myouga et al., 2008). The 3 isoforms of *Cu-ZnSOD* in *Arabidopsis* have different subcellular locations. *CSD1* is targeted to cytosol, *CSD2* is

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localized in chloroplasts, *CSD3* is present in peroxisomes, and *Mn-SOD* is localized to mitochondria (Kliebenstein et al., 1998).

Arabidopsis thaliana shows chilling tolerance features, while there are several mutants of this plant with the *chs* phenotype. These mutants have been placed into 4 classes based on obvious phenotypes following a 3-day exposure to chilling temperature (13 °C). Mutants in class 1 (*chs1–3*) first turn chlorotic, wilt, and eventually die. These *chs1* mutants died after 3 days of exposure to chilling temperatures and could not be rescued upon returning them to the normal growth temperature. The mature leaves of class 2 mutants (*chs4*) turn chlorotic, wilt, and die after chilling, whereas younger leaves of the rosette are unaffected. In class 3 *chs* mutants (*chs5,6*), leaves develop yellow patches, while their veins remain green. In class 4 mutants (*chs7–15*), the part of the leaf near the center of the rosette turns yellow. Class 2, 3, and 4 mutants finally flower and set seed after several weeks at chilling temperatures (Schneider et al., 1995a; Provart et al., 2003; Zhu and Provart, 2003; Zoldan et al., 2012). The expression pattern of the mutants upon chilling suggests that the normal function of the mutated loci prevents the widespread damaging effect of chilling on transcriptional regulation (Provart et al., 2003). Due to a limited number of studies related to the antioxidative system of these mutant lines of *A. thaliana*, this study investigates the expression patterns of some of the antioxidant enzymes in this plant. The role of antioxidative defense systems in plant responses to other abiotic stresses, such as drought, have been comprehensively documented (Sekmen et al., 2012; Saeidnejad et al., 2013). The reason for taking this approach is the phenotype of the mutant plants under chilling stress, namely, the yellow color of the aerial parts. This change in color likely arose from a defect in the photosynthetic system. Such an assumption would entail perturbations in the chloroplast, including its enzymatic defense mechanisms (FSDs). In addition, the role of mitochondria in response to stress was studied by inspecting the expression of the mitochondria-localized *MSD1*.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of EMS-mutagenized M2 populations of *chs1-1* (cs3097), *chs1-2* (cs6252), *chs2-1* (cs6298), *chs2-2* (cs6299) (Schneider et al., 1995a), and WT *Arabidopsis* ecotype Columbia were obtained from the European *Arabidopsis* Stock Center (NASC). Seeds were germinated in Metro-Mix soil (Scotts-Sierra Horticultural Products Co., Marysville, OH, USA) in flats and grown in GC-300TLH chambers (Conviron, Winnipeg and Korea) at 23 °C under a 16 h/8 h light/dark regime and 75% humidity. Plants received a light intensity of 350 mol s^{-1} m^{-2} through 8 bulbs emitting 7000 lm/m^2. In the 4th week, one-third of the mutant and WT plants were transferred to another growth chamber at 13 °C (chilling treatment) for 1 week, while one-third were transferred to a growth chamber set at 4 °C (in the present study 4 °C is considered cold treatment) (Shinozaki and Yamaguchi-Shinozaki, 2000; Thomashow, 2001; Fowler and Thomashow, 2002; Provart et al., 2003; Zoldan et al., 2012) for 1 week before samples were harvested. Other than the temperature, conditions were identical in all 3 chambers. Samples from the aerial parts of 10 different plants in each of the 3 groups were removed and pooled (Figure 1).

2.2. RT-PCR analysis

Total RNA was extracted from the shoots using QIAGEN RNeasy Plant Mini Kit (cat. no. 74904) according to the manufacturer's instructions. First strand cDNA was synthesized from total RNA using Accu Power RT/PCR Pre-Mix. The relative levels of *Mn-SOD1* (AT3G10920.1), *Fe-SOD2* (AT5G51100.1), and *Fe-SOD3* (AT5G23310) were measured by RT-PCR (R Corbett, CG1-96-Australia) using the *Arabidopsis* 18S rRNA (NM-S94466) as an internal standard. Second strand cDNA synthesis was performed at 42 °C for 30 min, and PCR amplification with *Mn-SOD* (NM_111929.3), *Fe-SOD2* (NM_124489.2), and *Fe-SOD3* (NM_122237.3) gene-specific primers (see Table 1 for primer sequences, product size, and melting temperatures) was carried out under the following conditions: 40 cycles of denaturation at 95 °C for 30 s; annealing at 63 °C (*MSD1*), 53 °C (*FSD2*), 53 °C (*FSD3*), and 54 °C (18S) for 1 min; and extension at 72 °C for 1 min. The amplification of the internal standard 18S rRNA cDNA was carried out in a similar manner using two 18S rRNA-specific primers. RT-PCR product was separated on a 1% agarose gel and visualized by ethidium bromide staining. The expected sizes of RT-PCR products of *Mn-SOD1* (*AtMSD1*), *Fe-SOD2* (*AtFSD2*), *Fe-SOD3* (*AtFSD3*), and 18S rRNA genes were 108, 242, 157, and 154 bp, respectively. Note that the PCR reactions contained both primer sets (i.e. 18S and the SOD genes) and loaded in a single lane of the 1% agarose gel, except in the case of *Fe-SOD3*. This PCR reaction only included the primer set for the *Fe-SOD3* gene, as the amplicons were nearly of equal size (i.e. 154 bp and 157 bp for 18S and *Fe-SOD3*, respectively), resulting in an overlap of the bands in the gel.

2.3. Concentration measurements of RNAs

Following mRNA extraction, concentrations were determined spectrophotometrically (T80$^+$ PG Instruments, UK). The absorbencies at 260 and 280 nm were determined, and the value of A260/280 was calculated. The A260 value was used in the formula C = OD × 20.

2.4. Primer design

Mn-SOD and *Fe-SOD* gene sequences were obtained from Tair (www.arabidopsis.info), and primers were

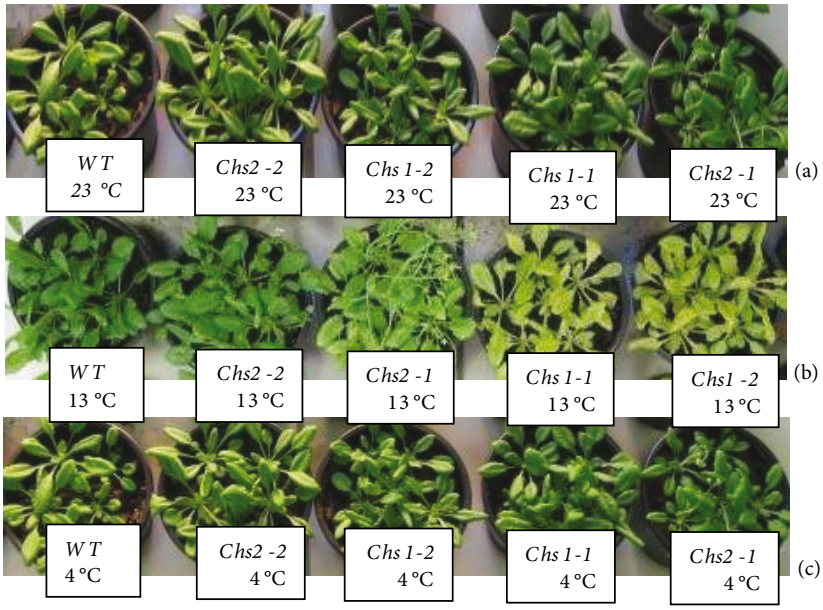


Figure 1. Phenotypes of WT, *chs1*, and *chs2* plants following transfer to chilling and cold conditions. Plants were grown for 4 weeks at 23 °C (a) and then transferred to a chilling temperature of 13 °C (b) or a cold temperature of 4 °C (c).

Table 1. Primer sequences used for RT-PCR and the product sizes.

Primer name	Sequence	Product size	Tm
18s-1L	CCTCCAATGGATCCTCGTTA	154	54
18s-1R	AAACGGCTACCACATCCAAG	154	54
FeSOD2-1L	ACTCCCAATGCTGTGAATCC	242	53.8
FeSOD2-1R	CTTCGGTGATGCAGAACTCA	242	53.4
FeSOD3-1L	GGATGTGTGGGAGCACTCTT	157	53.3
FeSOD3-1R	GATTGGGATGTTGGGTTCAC	157	54.1
MnSOD-1L	ACCCTAGCCGGCTTGAAGGAGAC	108	62.7
MnSOD-1R	GGCCGGTTCCAATGCGCCATA	108	64.3

designed using software available from the University of Massachusetts Medical School (http://biotools.umassmed.edu/bioapps/primer3). The extracted primer sequences were BLAST-analyzed at NCBI to ascertain their gene-specific nature. These primers were then purchased from BIONEER (Korea).

2.5. Determination of PSII photosynthetic efficiency

Leaves of seedlings from 5 individual plants (WT and 4 *chs* mutant plants) per treatment were used for chlorophyll (Chl) fluorescence analysis. Prior to fluorescence measurement, the abaxial surface of a circular piece of the excised leaf (1 cm diameter) was dark-adapted for 5 min using a dark leaf clip. The PSII photosynthetic efficiency (Fv/Fm) of leaves was estimated using a portable Chl fluorescence meter (Handy PEA, Hansatech, UK). PSII efficiency represents the maximal yield of the photochemical reaction in PSII.

2.6. Chlorophyll content

The relative Chl content was estimated by CL-01 Chl Content Meter (CL-01, Hansatech, Kings Lynn, UK) which determines the relative content. This equipment allowed us to compare plant responses to low temperature conditions.

2.7. Statistical analysis

Data presented are means ± standard error for 3 replicates. The data means were compared using Duncan's multiple range test with significance at $P < 0.001$.

3. Results

3.1. Photosynthetic efficiency

In the present work, the protective mechanisms were examined by comparing the *Arabidopsis* chilling sensitive lines with wild-type (WT) plants. These results indicated that under chilling temperature the Fv/Fm values of

chs mutant leaves, in particular *chs1* mutants, decrease significantly ($P < 0.001$) compared to both WT and cold-treated plants (Table 2). When these mutants were cold-treated they had a lower Fv/Fm value than plants treated with the normal temperature, but a higher value than plants at chilling temperature.

3.2. Chlorophyll content

The *chs* mutants had lower relative Chl contents than WT plants under chilling temperature. The lowest amount of relative Chl content was observed in *chs1* mutants (Table 3).

3.3. Expression pattern of AtMSD1 gene in leaves under low temperature stress

The expression pattern of *AtMSD1* gene in the leaves of *Arabidopsis* under low and normal temperatures was investigated by RT-PCR. The results of RT-PCR analysis showed that *AtMSD1* in *chs* mutants responded to chilling stress (Figure 2).

3.4. Expression patterns of AtFSD2 and AtFSD3 genes in leaves under low temperature stress

No PCR products of the expected size (≈242 and 157 bp, respectively, for *FSD2* and *FSD3*) could be amplified from mutant lines under low temperature stress, whereas the expression of *FSD2* and *FSD3* genes was observed in the WT plants (Figures 3).

4. Discussion

Symptoms of chilling injury, which include a reduction in photosynthetic capacity, wilting, development of chlorosis, and a cessation of growth in all *chs* mutants, become dominant one week after the transfer to chilling temperatures. The obvious phenotype and the results of the experiments have revealed that *chs1* mutants are very sensitive to chilling temperatures (Schneider et al., 1995a; Zoldan et al., 2012).

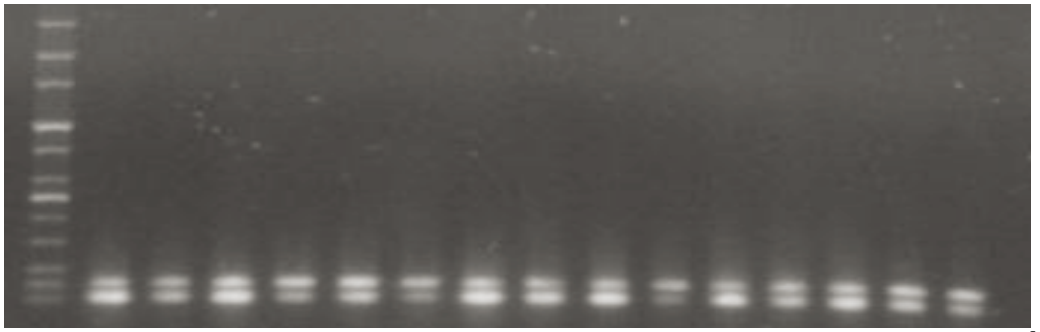
A well-known response of *chs* plants to low temperatures is a reduction in photosynthesis (Yadegari et al., 2007). The chilling-tolerant genotypes of maize (*Zea mays* L.), when compared to those of sensitive lines, display higher photosynthetic activity, higher content of total Chl, higher Chl *a:b* ratio, and a larger total carotenoid pool size, as well as a different carotenoid composition (Haldimann, 1999). Chl degradation has also been observed in *chs* maize (*Zea mays*, variety Penjalinan) exposed to low temperature stress (Aroca et al., 2001). A decrease in the Chl *a:b* ratio and total Chl content of 2 genotypes of pepper subjected to chilling treatment has also been reported (Koç et al., 2000). When the seedlings of 2 rice cultivars, IR8 (low-temperature sensitive) and Somewake (low-temperature tolerant), were exposed to chilling treatment (15 °C) the normal increase in Chl content of the developing leaf blade completely ceased (Maruyama et al., 1990). The observed

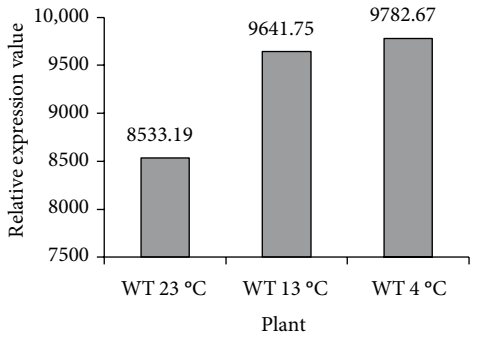
Table 2. Effect of chilling and cold stress on Fv/Fm of WT and *chs* mutants of *Arabidopsis* seedlings. Values are means ± SE of 3 separate measurements. Identical letters (a, ab, or c) show lack of significant difference.

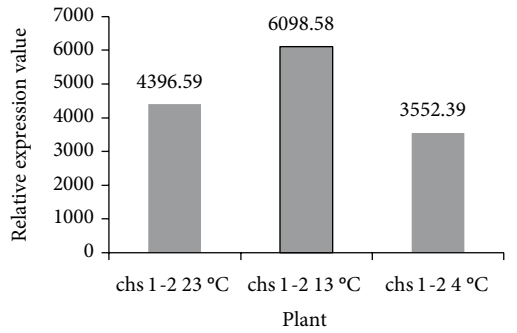
Plant genotype	Control	Chilling stress	Cold stress
WT	0.844 ± 0.003^{a}	0.844 ± 0.001^{a}	0.833 ± 0.002^{a}
Chs2-2	0.839 ± 0.002^{a}	0.816 ± 0.006^{a}	0.824 ± 0.001^{a}
Chs2-1	0.835 ± 0.001^{a}	0.813 ± 0.001^{ab}	0.827 ± 0.004^{a}
Chs1-2	0.833 ± 0.003^{a}	0.779 ± 0.018^{ab}	0.826 ± 0.012^{a}
Chs1-1	0.837 ± 0.002^{a}	0.728 ± 0.04^{c}	0.835 ± 0.001^{a}

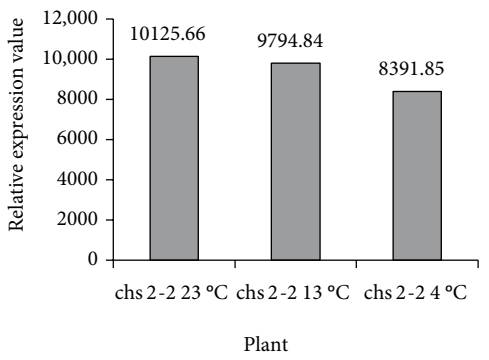
Table 3. Changes in the content of relative *chl* in the leaves of *Arabidopsis* plants subjected to chilling and cold stresses (4 °C). Values are means ± SE of 3 separate measurements. Identical letters (a, ab, or c) show lack of significant difference.

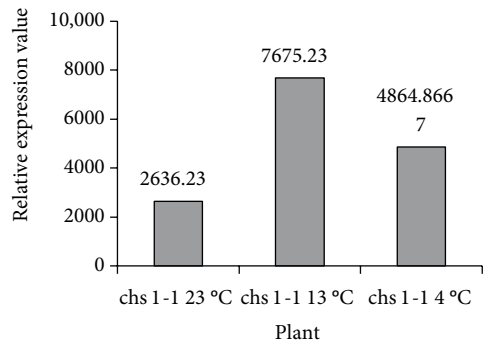
Plant genotype	Control	Chilling stress	Cold stress
WT	5.49 ± 0.151^{b}	4.38 ± 0.08^{de}	4.73 ± 0.108^{cd}
Chs2-2	6.36 ± 0.265^{a}	3.61 ± 0.197^{f}	5.24 ± 0.215^{b}
Chs2-1	5.12 ± 0.125^{bc}	3.16 ± 0.072^{f}	4.18 ± 0.128^{e}
Chs1-2	5.37 ± 0.12^{b}	2.11 ± 0.025^{g}	5.28 ± 0.135^{b}
Chs1-1	5.42 ± 0.213^{b}	2.04 ± 0.093^{g}	4.53 ± 0.108^{de}











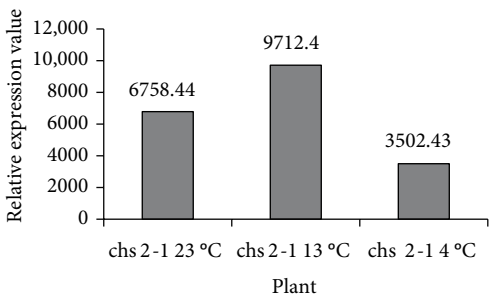


Figure 2. Reverse transcriptase PCR analysis of AT3G10920.1 (*Mn-SOD*) expression in *chs2-2*, *chs2-1*, *chs1-2*, *chs1-1*, and WT *Arabidopsis* leaves. Total RNA was extracted from WT and chilling sensitive mutant seedlings grown under control, chilling, and cold stress conditions. Primers for AT2G01010.1 (18S rRNA) amplification were used as an amplification control. PCR reactions were repeated twice with essentially identical results. Graphs drawn using Total Lab v. 1.10 software, and show expression rates quantitatively. The X-axis presents the different plants, while the Y-axis shows the relative expression value. Data was normalized according to 18S rRNA; M = marker.

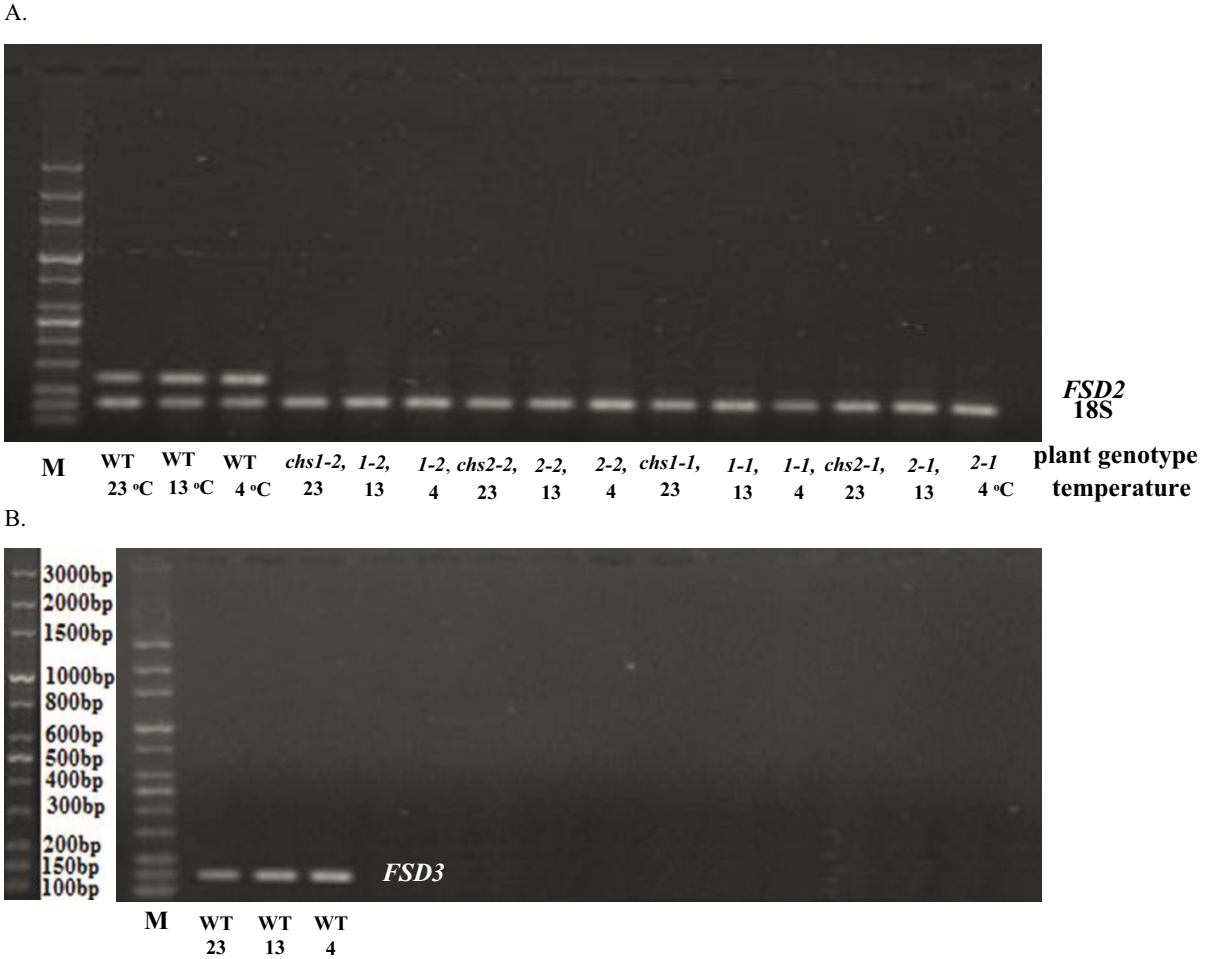


Figure 3. Reverse transcriptase PCR analysis of *FSD2* and *FSD3* expression in *chs2-2*, *chs2-1*, *chs1-2*, *chs1-1*, and WT *Arabidopsis* leaves. A. *Fe-SOD2*; B. *Fe-SOD3*; RT-PCR in WT plants: 1–23: WT at 23 °C, 1–13: WT at 13 °C, and 1–4: WT at 4 °C. Note that as the amplicons of the 18S and *Fe-SOD3* PCR reactions are almost of equal size (i.e. 154 bp and 157 bp, respectively) and their bands overlap in the gel, no primers for the 18S were included in these reactions; M = marker.

decline in Chl content induced by low temperature is in agreement with our results. Relative Chl content of our *chs* mutant plants decreased significantly under cold stress and more so under chilling. This reduction was more severe in *chs1* than in *chs2* mutants. Photosynthesis reduction due to low temperature is a well-known response of cold-sensitive plants (Yadegari et al., 2007). Our results in leaf Chl content revealed a slowdown of photosynthesis and lower Chl content in chilled mutant plants. This reduction may have resulted from a lower expression of the light harvesting complex protein (LHCP) components in photosystems I and II, lower activity of the oxygen forming complex, and lower electron transport for oxygen production, which eventually leads to the formation of ROS, mainly O_2^-. This increase in ROS may have, in turn, negatively affected plant growth and development. In addition, a reduction in Chl content may be a typical symptom of oxidative stress. Thus, the inability of *chs* mutants to form a capable photosynthetic apparatus at chilling temperature is likely to be an important factor in the cessation of their growth.

The Fv/Fm ratio is generally used to give an estimate of the basal efficiency of the PSII photochemical process (Björkman and Demming, 1987). Our results demonstrated that PSII was severely injured in *chs* mutants under the chilling condition. The Fv/Fm ratio decreased in *chs* mutants after 7 days at cold and chilling treatments; however, WT plants appeared to be less affected. In our results, *chs1* mutants showed a greater reduction in both Chl content and Fv/Fm value than *chs2* mutants at chilling. This finding is in line with the greater development of chlorosis detected in *chs1* mutants, which results in a susceptibility that is greater than in *chs2* mutants. This investigation did not measure the speed of net photosynthesis; however, fluorescence and maximum quantum efficiency may indirectly reflect photosynthetic activity. On this basis, our results revealed a greater reduction in PSII photochemical

efficiency in leaves under cold treatment compared to both the cold and control treatments, reflecting a reduction in the photosynthetic efficiency and possibly a photoinhibitory effect in the mutant plants. Recent studies have also revealed that exposure to low temperatures results in defects in chloroplast maintenance, including disruption of chloroplast protein accumulation (Schneider et al., 1995b). Studies have also found that the function of the *chs1* gene product may be required to maintain chloroplast function at low temperatures in *chs* mutants of *Arabidopsis* plants (Zoldan et al., 2012). These results suggested that *chs* mutants suffered a higher degree of oxidative damage induced by chilling and cold due to excessive ROS production, a response consistent with other reports (Saxby et al., 2003; Zhou et al., 2006).

The expression of *MSD1* increased in the *chs1* mutants under chilling conditions. This response is similar to that observed for *MSD1* gene in the germinated embryonic axes of *Nelumbo nucifera* in a strong response to both chilling and oxidative stress (Li et al., 2009), and to the increased expression of *Mn-SOD* genes in both *Candida albicans* cells (Raychaudhuri and Deng, 2000) and the leaves of cucumber (*Cucumis sativus* L.) (Lee and Lee, 2000). The presence of transgenic *Mn-SOD* in maize plants had clear effects on foliar tolerance to chilling and oxidative stress (Van Breusegem et al., 1999b). When exposed to cold, *chs* maize showed lower SOD activity than the chilling-tolerant maize (Pinhero et al., 1997). Transcripts for *Mn-SOD* in WT tobacco increased during chilling stress (Tsang et al., 1991), and *Mn-SOD* from tea, TEENALI, exhibited a role in low temperature tolerance in relation to dormancy (Vyas and Kumar, 2005). While *MSD1* expression increased under chilling treatment in *chs1* mutants, its expression decreased under cold conditions, leading us to speculate on the existence of a different pathway for the expression of *MSD1* under cold and chilling conditions. Taken together, it appears that the increased expression of *MSD1* is indicative of its role in protection against chilling and, presumably, the proper function of the mitochondrion during chilling stress in *chs* mutants.

There are 3 isoforms of iron superoxide dismutases in *Arabidopsis thaliana*: Fe superoxide dismutase1 (*FSD1*), *FSD2*, and *FSD3*. *FSD2* and *FSD3* play essential roles in early chloroplast development and the leaves of transgenic *Arabidopsis* plants expressing *FSD2* and *FSD3* individually and in combination, and they show lower inactivation of PSII, as indicated by Fv/Fm, than do WT plants (Myouga et al., 2008). During in-vitro–generated oxidative stress, PSII was better protected in transgenic tobacco plants overproducing *Arabidopsis thaliana Fe-SOD* (Van Camp et al., 1996). Furthermore, a mutant strain lacking *Fe-SOD* in the cyanobacterium *Synechococcus* is sensitive to photo-oxidative stress, and its PSI and II complexes are damaged (Herbert et al., 1992). In WT tobacco, mRNA levels for the chloroplastic *Fe-SOD* increased moderately

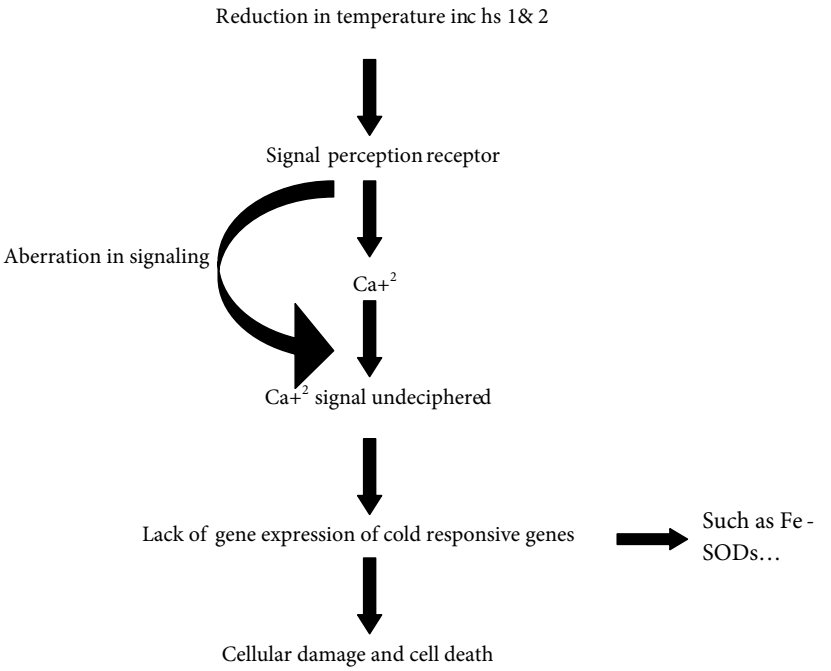


Figure 4. Model for calcium regulation of cold responsive genes (*cor*) expression in *chs1* and *chs2* mutants.

during chilling stress (Hérouart et al., 1991), while PSII was severely impaired in transgenic tobacco plants with severely reduced *Fe-SOD* levels (Zhang et al., 2011). It has been reported that the Pro-P5C cycle resulting from the incomplete degradation of proline in cold sensitive mutants leads to the formation of increased levels of ROS (Khavarinejad et al., 2013). Hydrogen peroxide, as a member of ROS, is an inhibitor of FSDs. Therefore, it is very likely that a lack of *FSD* expression is somehow related to increased levels of hydrogen peroxide. The lack of expression of the *FSD* genes in mutant plants is likely due to a perturbation in the calcium signaling pathway. It has been reported that in the *Arabidopsis chs3* group 1 mutants, cold signaling is regulated through the calcium signaling pathway, which slows down in these mutants during chilling treatment (Knight, 2002). Such a scenario may also exist in our mutant plants (*chs1* and *chs2*). Since the genes involved in the antioxidant pathway are also the components of cold response genes (CORs), the expression of the genes in the antioxidant pathway is dependent on the transfer of the appropriate signal from this pathway. We can conclude that a reduction in the transfer of calcium may have led to a lack of expression of *Fe-SOD* genes (Figure 4). Therefore, according to these reports and our results, the expression of *Fe-SOD* genes is important for the protection of chloroplasts against oxidative and chilling stress, and probably the chloroplast organelle is damaged under chilling condition in these mutants. In summary, the decrease in Fv/Fm and Chl content in *chs* mutants due to chilling stress and a lack of expression of *Fe-SOD* genes are all indicative of a possible defect in chloroplast function. Furthermore, an increase in the expression of *MSD1* gene in *chs1* chilled mutants suggests an important role for *Mn-SOD* in the protection against chilling and oxidative stress as well as a lack of defect in the action of mitochondria in chs mutants under chilling temperatures.

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